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(21) International Application Number: PCT/AU98/00648 (22) International Filing Date: 14 August 1998 (14.08.98) (30) Priority Data: PO 8560 14 August 1997 (14.08.97) AU (71) Applicants (for all designated States except US): COMMON-WEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANISATION [AU/AU]; Limestone Avenue, Campbell, ACT 2601 (AU). PIG RESEARCH DEVELOPMENT CORPORATION COMPUTER ASSOCIATE HOUSE [AU/AU]; 10 National Circuit, Barton, ACT 2600 (AU). (72) Inventors; and (75) Inventors/Applicants (for US only): JOHNSON, Michael, Anthony [AU/AU]; 46 Harold Street, Thornbury, VIC 3071 (AU). HAMMOND, Jeffrey, Michael [GB/AU]; 5 Cantala Drive, Jan Juc, VIC 3228 (AU). (74) Agent: WATERMARK PATENT & TRADEMARK ATTORNEYS; 2nd floor, 290 Burwood Road, Hawthorn, VIC 3122 (AU).		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published With international search report.	
(54) Title: RECOMBINANT PORCINE ADENOVIRUS VECTOR			
(57) Abstract			
<p>This invention relates to a recombinant vector including a recombinant porcine adenovirus, stably incorporating and capable of expression of at least one heterologous nucleotide sequence. The nucleotide sequence is preferably one which encodes an antigenic determinant of Hog Cholera Virus or Pseudorabies virus. The further invention relates to a method of production of recombinant vectors, to methods of preparation of vaccines based on the vectors, to administration strategies and to methods of protecting pigs from disease.</p>			

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RECOMBINANT PORCINE ADENOVIRUS VECTOR

FIELD OF INVENTION

This invention relates to delivery vectors for antigen producing genes (heterologous gene sequences or fragments thereof) used to generate immune
5 responses in commercial pigs susceptible to decimation by disease. Such vectors are especially useful for the preparation of vaccines which can be easily administered on a large scale to protect pigs against disease. This invention also relates to a method of production of suitable delivery vectors, to methods of preparation of vaccines based on the vectors, to administration strategies and to
10 a method protecting pigs from disease.

BACKGROUND

The productivity of the intensive pig industry depends on the control of infectious diseases. Whilst diseases can be controlled in part by good hygiene and quarantine measures, the industry must still rely on vaccination to protect
15 herds. In a commercial situation, the cost per animal is high in terms of feed and current disease control costs and therefore, the costs in disease prevention and control by any newly proposed vaccine must be cheap, effective and easy to deliver.

Conventionally, vaccines constituting live viral particles have been
20 prepared by virus passage and selection of attenuated forms. Alternatively, killed vaccines were prepared from virulent viruses.

The most recent description of the use of viral vectors in the control of disease in pigs was the deletion mutant of pseudorabies virus for the control of Aujeszky's disease. The use of a herpesvirus as a vector has the advantage of
25 being able to stimulate a humoral and cell-mediated response, thus providing possible life long protection. Another advantage is the ability to insert other heterologous sequences in this vector, being expressed from a suitable promoter, to produce antigens for exposure to the animals immune system, thus protecting against two diseases. There are disadvantages of this system.
30 Firstly, there is the issue of latency. Herpesviruses have the ability to intergrate into the neurons in ganglia for the life of the animal. It only requires a suitable stress on the animal to cause the reactivation of the virus and consequently full

disease. However, it is now known that the deletion of a specific gene, glycoprotein E, will attenuate the virus and prevent reactivation from latency. Therefore, this deletion vector is now widely used as an eradication vector for Aujeszky's disease and subsequently will not be available as a suitable vector for
5 the delivery of other antigens.

It is thus the aim of this invention to provide a delivery vehicle for heterologous sequences of genetic material that is particularly suited to administration on a large scale.

In particular, it is the aim of this invention to provide or enhance means for
10 generation and/or optimisation of antibodies or cell-mediated immunity so as to provide protection against infection with common porcine diseases. It is an additional aim to provide a process for preparation of a suitable means for generation and/or optimisation of antibodies or cell-mediated immunity so as to protect pigs against infection with common porcine diseases. It is a further aim
15 to provide a protection strategy.

SUMMARY OF INVENTION

The invention provides, in one embodiment, a recombinant porcine adenovirus capable of expressing DNA of interest, said DNA of interest being stably integrated into an appropriate site of said recombinant porcine
20 adenovirus genome.

In another embodiment the invention provides a recombinant vector including a recombinant porcine adenovirus which stably incorporates at least one heterologous nucleotide sequence. Preferably the heterologous nucleotide sequence is capable of expression as an antigenic polypeptide. The antigenic
25 polypeptide encoded by at least one nucleotide sequence is preferably foreign to the host vector.

In a further embodiment of the present invention the heterologous nucleotide sequence is capable of expression as an immuno-potentiator molecule.

30 It is also to be understood that the heterologous nucleotide sequence may encode for and/or express, an antigenic polypeptide and an immuno-potentiator molecule.

The recombinant vector may comprise a live recombinant porcine adenovirus in which the virion structural proteins are unchanged from those in the native porcine adenovirus from which the recombinant porcine adenovirus is produced.

- 5 This invention is partially predicated on the discovery that there are non-essential regions in the porcine adenovirus genome which do not correspond to those characterised previously on other adenoviruses thus making this virus particularly suited to delivery of heterologous sequences.

This invention is also predicated on the discovery that the porcine
10 adenovirus generates a prolonged response in pigs thus making it well suited as a vaccine vehicle. Furthermore, the existence of a number of serotypes specific to respiratory or gastrointestinal tracts, allows the selection of a vaccine vehicle suited to a target organ and the type of immune response required.

The invention is also predicated on the discovery that porcine adenovirus
15 can package genomic DNA greater than the 105% rule for mammalian adenoviruses with intermediate size genomes and that the resultant packaged virions are stable in vitro and in vivo.

Adenoviruses are a large and diverse family, having been isolated from many living species, including man and other mammals as well as a variety of
20 birds. As a result adenoviruses have been separated into at least two genera, the *Mastoadenoviridae* and the *Aviadenoviridae*, and more recently a third genera has been proposed, the *Atadenoviridae*, which includes some bovine and avian adenoviruses (egg drop syndrome) (Benkö and Harrach, Archives of Virology 143, 829-837, 1998).

- 25 Porcine adenoviruses are prevalent infectious agents of pigs and to date four distinct serotypes have been recognised (Adair and McFerran, 1976) and evidence for at least one more (Derbyshire *et al.*, 1975). Of the four serotypes found, three (serotypes 1 to 3) were isolated from the gastrointestinal tract while the fourth was recovered from the respiratory system. The porcine adenoviruses
30 are considered to be a low pathogenic widespread agent and although isolations were made in general from diseased animals, it was most likely that the adenovirus was present only as a secondary infection. They have been

4.

isolated from pigs with diarrhoea and respiratory infections but it has been considered that at least the gastrointestinal adenovirus infections are usually asymptomatic (Sanford and Hoover, 1983). Porcine adenoviruses are spread by ingestion or inhalation and experimental infection via oral, intranasal and
5 intratracheal inoculations have resulted in uptake of the virus. Experimental pathogenicity studies have shown that the primary sites of infection are the lower small intestine probably the tonsil (Sharpe and Jessett, 1967; Shadduck *et al.*, 1968). With serotype 4 infection, a viraemia appears to develop in experimental infections. However, this may be a less common manifestation
10 with the gastrointestinal serotypes (Shadduck *et al.*, 1968). Faecal excretion is the most common cause for spread of PAV, being present for several weeks post infection. Nasal shedding also occurs under experimental conditions. PAV's role in pneumonia has been suggested to be that of either a predisposing factor or a synergist (Kasza *et al.*, 1969; Schiefer *et al.*, 1974) but experimental
15 pneumonia with serotype 4 did not require a second agent to produce disease (Smith *et al.*, 1973).

Porcine adenoviruses have yet to be examined in much detail and little is known about their role in disease or how common they are. This is due to the fact that they do not produce any significant disease in herds and have failed to
20 draw the interest of industry through loss of production. It is likely that the number of serotypes of porcine adenoviruses is much greater than four and that it probably exists in the majority of pig herds as a normal commensal.

Work done on porcine adenovirus in regards to its morphology and molecular biology, has shown some similarities with other *Mastadenoviruses*
25 examined. Its morphology is that of other adenoviruses examined with an icosahedral capsid containing a core of a double stranded DNA genome. Very little work on the characterisation of the PAV genome has been published (Benkő *et al.*, 1990, Kleiboeker *et al.*, 1993, Reddy *et al.*, 1993, Kleiboeker, 1994). The size of the PAV genome (approx. 34.8 kb) is slightly smaller than
30 that of human adenoviruses (approx. 35.9 kb). One study has shown using hybridisation with DNA probes from the total genome of human adenovirus type 2 that there is reasonable DNA homology between the porcine and human

adenoviruses (Benkő *et al.*, 1990). A recent report on the serotype 4 PAV demonstrated that its genomic layout was also similar to that of the human adenoviruses in the area of the L4 and E3 regions (including the 33K and pVIII genes) even though the sequence homology was not as strong as may have
5 been expected (Kleiboeker, 1994).

While choosing appropriate PAV for development as a live vectors to deliver vaccines to pigs, it is important to take into account the natural prevalence of serotypes. Those serotypes not commonly encountered in the field have an obvious advantages over those to which pigs are frequently
10 exposed and to which they may have developed immunity.

A further consideration is the ability of the vector to remain active in the pig beyond the period which maternal antibodies in colostrum protect pigs immediately post-birth.

Other important considerations in choosing potential PAV vectors are
15 pathogenicity and immunogenicity. Preferably live vector viruses should be highly infectious but non-pathogenic (or at least attenuated) such that they do not themselves adversely affect the target species.

The preferred candidates for vaccine vectors are non-pathogenic isolates of serotype 4 (respiratory) and serotype 3 (gastrointestinal). Serotype 3 has
20 been chosen as the serotype of choice due to excellent growth abilities in continuous pig kidney cell lines. The isolation of other serotypes, which seems likely, may well alter this selection. It is notable that the more virulent strains produce a greater antibody response.

Heterologous nucleotide sequences which may be incorporated into non-
25 essential regions of the viral genome and which may encode the antigenic determinants of infectious organisms against which the generation of antibodies or cell-mediated immunity is desirable may be those expressing antigenic determinants of intestinal infections caused by gastrointestinal viruses; for example rotavirus or parvovirus infections, or respiratory viruses, for example
30 parainfluenza virus, or that of Japanese encephalitis.

Heterologous nucleotide sequences which may be incorporated include the antigenic determinants of the agents of:

- Porcine parvovirus
- Mycoplasma hyopneumonia
- Porcine parainfluenza
- Transmissible gastroenteritis (porcine coronavirus)
- 5 Porcine rotavirus
- Hog cholera virus (Classical swine fever)
- Swine dysentery
- African swine fever virus
- Pseudorabies virus (Aujeszky's disease virus), in particular the
- 10 glycoprotein D of the pseudorabies virus
- Porcine respiratory and reproductive syndrome virus (PRRSV)
- Heterologous nucleotide sequences more preferred for incorporation in the vectors of the invention are those expressing antigenic determinants of porcine parvovirus, porcine rotavirus, porcine coronavirus and classical swine
- 15 fever virus.

It is also envisaged the heterologous sequences incorporated may be immuno-potentiator molecules such as cytokines or growth promoters, for example porcine interleukin 4 (IL4), gamma interferon (γ IFN), granulocyte macrophage colony stimulating factor (GM-CSF), granulocyte colony stimulating

20 factor (G-CSF), FLT-3 ligand and interleukin 3 (IL-3).

The type of immune response stimulated by candidate vectors may affect the selection of heterologous nucleotide sequences for insertion therein. PAV serotypes 1, 2 and 3, which naturally infect via the gut may induce local mucosal immunity and are thus more suitable for infections of the intestines (eg classical

25 swine fever virus). PAV serotype 4, which naturally infects via the respiratory system, may be more suitable for infections of the respiratory tract (eg porcine parainfluenza) may also induce good local immunity.

The DNA of interest which may comprise heterologous genes coding for antigenic determinants or immuno-potentiator molecules may be located in at

30 least one non-essential region of the viral genome.

Non-essential regions of the viral genome which may be suitable for the purposes of replacement with or insertion of heterologous nucleotide

sequences may be non-coding regions at the right terminal end of the genome at map units 97 to 99.5. Preferred non-coding regions include the early region (E3) of the PAV genome at map units 81-84.

The heterologous gene sequences may be associated with a promoter
5 and leader sequence in order that the nucleotide sequence may be expressed in situ as efficiently as possible. Preferably the heterologous gene sequence is associated with the porcine adenoviral major late promoter and splice leader sequence. The mammalian adenovirus major late promoter lies near 16-17 map units on the adenovirus genetic map and contains a classical TATA
10 sequence motif (Johnson, D.C., Ghosh-Chondhury, G., Smiley, J.R., Fallis, L. and Graham, F.L. (1988), Abundant expression of herpes simplex virus glycoprotein gB using an adenovirus vector. Virology 164, 1-14).

The splice leader sequence of the porcine adenovirus serotype under consideration is a tripartite sequence spliced to the 5' end of the mRNA of all
15 late genes.

The heterologous gene sequence may also be associated with a polyadenylation sequence.

Instead of the porcine adenoviral major late promoter, any other suitable eukaryotic promoter may be used. For example, those of SV40 virus,
20 cytomegalovirus (CMV) or human adenovirus may be used.

Processing and poly adenylation signals other than those of porcine adenoviruses may also be considered, for example, that of SV40.

In a further aspect of the invention there is provided a recombinant vaccine for generating and/or optimising antibodies or cell-mediated immunity
25 so as to provide or enhance protection against infection with an infectious organism in pigs, the vaccine including at least one recombinant porcine adenovirus vector stably incorporating at least one heterologous nucleotide sequence formulated with suitable carriers and excipients. Preferably the nucleotide sequence is capable of expression as an antigenic polypeptide or as
30 an immuno-potentiator molecule. More preferably, the heterologous nucleotide sequence may encode for and/or express, an antigenic polypeptide and an immuno-potentiator molecule.

The antigenic polypeptide encoded by the at least one nucleotide sequence is preferably foreign to the host vector. At least one nucleotide sequence may be associated with a promoter/leader and a poly A sequence.

The recombinant vaccine may include live recombinant porcine
5 adenovirus vector in which the virion structural proteins are unchanged from that in the native porcine adenovirus from which the recombinant porcine adenovirus is produced.

Preferred vector candidates for use in the recombinant vaccine are PAV
isolates of serotype 3 and 4. Use of other serotypes is possible, depending on
10 herd existing immunity and its environment.

The vaccine may be directed against respiratory and intestinal infections caused by a variety of agents. In order to direct the vaccine against a specific infectious organism, heterologous gene sequences encoding the antigenic determinants of those infectious organisms may be incorporated into non-
15 essential regions of the genome of the porcine adenovirus comprising the vector. If the vaccine is to be used to optimise protection against disease, suitable heterologous nucleotide sequences may be those of immunopotentiators such as cytokines or growth promoters.

The vaccine may comprise other constituents, such as stabilisers,
20 excipients, other pharmaceutically acceptable compounds or any other antigen or part thereof. The vaccine may be in the form of a lyophilised preparation or as a suspension, all of which are common in the field of vaccine production.

A suitable carrier for such as a vaccine may be isotonic buffered saline.

In a further aspect of the invention, there is provided a method of
25 preparing a vaccine for generation and/or optimisation of antibodies or cell-mediated immunity so as to induce or enhance protection against an infectious organism in a pig, which includes constructing a recombinant porcine adenovirus vector stably incorporating at least one heterologous nucleotide sequence, and placing said recombinant porcine adenovirus vector in a form
30 suitable for administration. Preferably the nucleotide sequence is capable of expression as an antigenic polypeptide although it may also be an immunopotentiator molecule. More preferably, the nucleotide sequence may encode

for and/or express, an antigenic polypeptide and an immuno-potentiator molecule. The nucleotide sequence is conveniently foreign to the host vector.

Even more preferably the nucleotide sequence is associated with
5 promoter/leader and poly A sequences.

The form of administration may be that of an enteric coated dosage unit, an inoculum for intra-peritoneal, intramuscular or subcutaneous administration, an aerosol spray, by oral or intranasal application. Administration in the drinking water or in feed pellets is also possible.

10 In another aspect of the invention, there is provided a method of producing a porcine adenovirus vaccine vector which includes inserting into a porcine adenovirus at least one heterologous nucleotide sequence. Said heterologous nucleotide sequence is preferably capable of expression as an antigenic polypeptide although it may also be an immuno-potentiator molecule.
15 More preferably, the nucleotide sequence may encode for and/or express, an antigenic polypeptide and an immuno-potentiator molecule.

Preferably the antigenic polypeptide encoded by the at least one nucleotide sequence is foreign to the host vector.

More preferably the heterologous nucleotide sequence is associated with
20 promoter/leader and poly A sequences.

In one method of construction of a suitable vector the non-essential region to be altered to incorporate foreign DNA could be constructed via homologous recombination. By this method the non-essential region is cloned and foreign DNA together with promoter, leader and poly adenylation
25 sequences is inserted preferably by homologous recombination between flanking sequences. By this method also, deletion of portions of the non-essential region is possible to create extra room for larger DNA inserts that are beyond the normal packing constraints of the virus.

By this method a DNA expression cassette containing an appropriate
30 PAV promoter with foreign gene sequence as well as leader sequences and poly adenylation recognition sequences can be constructed with the unique restriction enzyme sites flanking the cassette enabling easy insertion into the

PAV genome.

In another aspect of the invention there is provided strategies for administration of the vaccines of the invention.

In one strategy, a heterologous antigen and immuno-modulatory molecule such as a cytokine may be expressed in the same recombinant and delivered as a single vaccine.

In one strategy according to the invention PAV vector based vaccines may be administered as 'cocktails' comprising 2 or more virus vectors carrying different foreign genes or immuno-potentiators.

10 In a preferred vaccination strategy of the invention, the 'cocktail' or simultaneous strategy, a vaccine based on both PAV serotype 3 and serotype 4 is used.

In another preferred strategy, a base recombinant serotype 3 porcine adenovirus is constructed and the fiber gene from serotype 4 replacing that of serotype 3 or the fiber from serotype 4 additionally cloned into the vaccine to broaden the targeting of the invention to both gut and respiratory delivery.

In an alternative strategy according to the invention, PAV vector based vaccines may be administered consecutively of each other to either administer booster vaccines or new vaccines at some stage subsequent to initial PAV vaccination. The vaccines used are preferably based on heterologous PAV isolates.

In a preferred version of the "consecutive" strategy, vaccines based on isolates serotypically unrelated are selected so as to achieve maximum protection against infection. In one example of such a strategy a vaccine based on PAV serotype 3 is administered subsequently or prior to vaccination with a vaccine based on PAV serotype 4.

Pigs are conveniently inoculated with vector vaccines according to the invention at any age. Piglets may be vaccinated at 1 day old, breeders may be vaccinated regularly up to point of giving birth and thereafter.

30 Preferably according to either the consecutive strategy or the cocktail strategy, pigs are vaccinated while still not fully immunocompetent. More conveniently, day-old pigs can be vaccinated for protection against re-infection

after a period of 4 weeks subsequent to initial vaccination.

In a further embodiment of the invention there is provided a method for producing an immune response in a pig including administering to the pig an effective amount of a recombinant vaccine according to the invention. An effective amount is an amount sufficient to elicit an immune response, preferably at least 10^4 TCID₅₀ per dose.

The vaccine of the invention may of course be combined with vaccines against other viruses or organisms such as parvovirus or Aujeszky's disease at the time of its administration.

10 In a preferred aspect of this embodiment of the invention, administration is by oral delivery or intra-nasally.

Methods for construction and testing of recombinant vectors and vaccines according to this invention will be well known to those skilled in the art. Standard procedures for endonuclease digestion, ligation and electrophoresis were carried out in accordance with the manufacturer's or suppliers instructions. Standard techniques are not described in detail and will be well understood by persons skilled in the art.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates the DNA restriction endonuclease map of the entire PAV serotype 3 genome.

Figure 2 illustrates the sequence characterisation and cloning of the major later promoter and splice leader sequences of PAV serotype 3.

Figure 3 illustrates the sequences of the major later promoter, upstream enhancer sequence and splice leaders 1, 2 and 3.

25 Figure 4 illustrates the terminal 720 bases of the right end of the genome.

Figure 5 illustrates the promoter region of E3 and the overlapping L4 area.

Figure 6 illustrates a preferred method of construction of a PAV vector.

Figure 7 represents temperature data of pigs vaccinated with a PAV based vaccine following challenge with CSFV antigen.

30 Figure 8 graphically represents anti-PAV antibody levels detected by ELISA in pigs pre and post vaccination with a PAV based vaccine.

Figure 9 graphically illustrates the development of neutralising antibodies in pigs vaccinated with a PAV based vaccine pre and post challenge with CSFV antigen.

Figure 10 graphically illustrates the mean white blood cell (WBC) counts of pigs vaccinated with a recombinant PAV vaccine expressing porcine G-CSF.

Figure 11 graphically illustrates the percentage change in white blood cell (WBC) counts following vaccination with a recombinant PAV vaccine expressing porcine G-CSF.

Figure 12 graphically represents the percentage change in monocyte cell populations following vaccination with recombinant PAV-G-CSF.

Figure 13 graphically represents the percentage change in lymphocyte cell populations following vaccination with recombinant PAV-G-CSF.

Figure 14 graphically represents the change in stimulation of T-cells following vaccination with recombinant PAV-G-CSF.

15 PREFERRED EMBODIMENTS

Aspects of preferred embodiments of the invention based on PAV isolates serotype 3 and serotype 4 will now be described. Whilst these two isolates have been selected because of their sites of infection in the pig, it will be appreciated that other isolates of porcine adenovirus may be more suitable for construction of vaccine vectors provided the criteria for selection described herein before are met.

In general, PAV are considered of low pathogenicity with little consequence in the field. The pathogenic significance of PAV is reviewed in Derbyshire, 1989. The first report of isolation of PAV was from a 12 day old pig with diarrhoea (Haig *et al.*, 1964). Two years later, PAV type 4 was first reported, isolated from the brain of a pig suffering from encephalitis of unknown cause (Kasza, 1966). Later reports have associated PAV mainly with diarrhoea in the field although this is normally low grade. PAV can also be regularly isolated from healthy animals with no disease signs and it is quite likely that its isolation from diseased animals is more a coincidence of its prevalence than an indicator of pathogenicity. However, an association between serotype 4 and respiratory disease has been reported (Watt, 1978) and this has been supported

by experimental infection (Edington *et al.*, 1972). Experimental infections with gastrointestinal serotypes of the virus (eg serotype 3) have been able to produce diarrhoea but the pathological changes produced were not clinically significant.

- 5 The genome of the selected PAV serotype 3 was characterised by conventional methods. The DNA restriction endonuclease maps of the entire genome is illustrated in figure 1. The genomes are orientated left to right. By convention adenovirus genomes are normally orientated such that the terminal region from which no late mRNA transcripts are synthesised is located at the left
10 end. The enzymes used to generate the map are indicated at the edge of each map.

CHARACTERISATION OF MAJOR LATE PROMOTER (MLP) AND SPLICE
LEADER SEQUENCES (LS) OF PAV SEROTYPE 3

Identification and cloning of the PAV MLP

- 15 By use of restriction enzyme and genetic maps of the PAV serotype 3 genome, a region was located that contained the MLP and leader sequences (Fig 1). The fragments identified in this region were cloned into plasmid vectors and sequenced.

20 The MLP promoter sequence was identified as containing a classical TATA sequence, the only one in the region sequenced, as well as upstream factors and was subsequently confirmed by the location of the leader sequence and the transcriptional start site.

Figures 2 and 3 illustrate the sequence characterisation of the major late promoter and splice leader sequences of PAV serotype 3.

- 25 In order to determine the structure and sequence of the leader sequence spliced to late mRNA, porcine kidney cells were infected with PAV and the infection was allowed to proceed until late in the infection cycle (usually 20-24 hr p.i.). At this time total RNA was purified from the infected cells using the RNAgents total RNA purification kit (Promega). The isolated RNA was
30 precipitated with isopropanol and stored at -70°C in 200 µl aliquots until

required. Poly A (mRNA) was isolated from total RNA by the use of the Poly AT tract System (Promega, USA). The isolated mRNA was used in cDNA production.

For cDNA production, oligonucleotides were produced to the
5 complimentary strand of the hexon gene and the penton base gene, both being MLP transcripts. A further oligonucleotide was produced which covered the proposed cap site of the major late transcript, 24 bases downstream of the TATA box. This oligonucleotide was used in conjunction with that used in cDNA production in Taq polymerase chain reaction. The resulting DNA produced from
10 positive clones was digested with appropriate restriction enzymes to determine the size of the inserted fragment. DNA sequencing of these inserted fragments was performed using a modification of the chain termination technique (Sanger, F., Nicklen, S and Gulson, A.R., 1977, DNA sequencing with chain terminating inhibitors. PNAS USA 74: 5463-5467) so as to allow Taq DNA polymerase
15 extension (Promega, USA).

To confirm the leader sequence cap site, fresh cDNA was prepared and this time a tail of dGTP residues added to it. Briefly, cDNA was incubated with 1 mM dGTP and approximately 15 units of terminal deoxynucleotidyl transferase (Promega) in 2 mM CaCl₂ buffer at 37°C for 60 minutes. The reaction was
20 stopped by heating to 70°C for 10 minutes. The DNA was then ethanol precipitated and resuspended in a volume suitable for use in polymerase chain reaction (PCR). PCR was performed as previously described using a poly (dC) oligonucleotide with a *Xba*I site at the 5' end. Resulting fragments were blunt ended with T4 DNA polymerase at 37°C for 30 minutes in the presence of
25 excess nucleotides and cloned into the *Sma*I site of the pUC18 vector. DNA preparation and sequencing were performed, as described previously, on clones shown to be positive by hybridisation.

Figure 3 illustrates the separate sequences of the major late promoter, upstream enhancer sequence and splice leaders 1, 2 and 3 as determined from
30 cDNA studies. Figure 2 illustrates the DNA sequence of the complete promoter cassette with the components joined together.

CHARACTERISATION OF NON-ESSENTIAL REGIONS OF VIRAL GENOME

The right end was identified by cloning and complete sequencing of the PAV serotype 3 *ApaI* fragment J of approximately 1.8 Kbp. The inverted terminal repeat (ITR) has been determined by comparison of the RHE sequence with that of the left hand end. The ITR is 144 bases long and represents the starting point into which potential insertions can be made. Figure 4 shows the sequence of the terminal 720 bases. Restriction endonuclease sites of interest for insertion of foreign DNA are indicated in the terminal sequence. A putative TATA site for the E4 promoter is identified, this being the left most end for the possible site of insertion. Initial insertions will be made into the *SmaI* or *EcoRI* sites.

The E3 region of the genome, this also being a non-essential area, has been located and cloned. The promoter region of E3 has been identified and the overlapping L4 area sequenced (Figure 5). The region of the E3 after the polyadenylation signal of the L4 is also a possible site for insertion and can also be used for deletion to create more room for larger cassette insertions.

CONSTRUCTION OF PAV VECTOR

Figure 6 illustrates a preferred method of construction of a PAV vector. The right hand end *ApaI* fragment J of PAV serotype 3 is cloned and a unique *SmaI* restriction endonuclease site 230 bp from the inverted repeats was used as an insertion site.

The major late promoter expression cassette containing the E2 (gp55) gene of classical swine fever virus (hog cholera virus) was cloned into the *SmaI* site of the RHE fragment.

25. A preferred method of homologous recombination was cutting genomic PAV 3 DNA with *HpaI*, a unique site in the genome, and transfecting this DNA with *ApaI* cut expression cassette plasmid containing gp55.

The DNA mix was transfected into preferably primary pig kidney cells by standard calcium chloride precipitation techniques.

30. The preferred method of transfection generates recombinant virus through homologous recombination between genomic PAV 3 and plasmid (Fig 6).

DETAILED DESCRIPTION OF THE INVENTIONCONSTRUCTION OF PAV VECTOR

The following examples show the construction of representative recombinant porcine adenoviruses of this invention. The recombinant viruses were
5 propagated and titred on primary porcine kidney cells.

1 Construction of PAV-gp55

An expression cassette consisting of the porcine adenovirus major late promoter, the classical swine fever virus (CSFV) gene (gp55) and SV 40 polyA was inserted into the *Sma*I site of the right hand end (MU 97-99.5) of porcine
10 adenovirus serotype 3 and used to generate in porcine primary kidney cells a recombinant PAV 3. The size of the expression cassette was 2.38 kilobase pairs. No deletion of the genomic PAV 3 was made. Mammalian adenoviruses with intermediate genomes (~36kb) have been shown to accommodate up to 105% of the wild-type genomic length, and genomes larger than this size are
15 either unpackageable or extremely unstable, frequently undergoing DNA rearrangements (Betts, Prevec and Graham, Journal of Virology 67, 5911-5921 (1993), Packaging capacity and stability of human adenovirus type 5 vectors: Parks and Graham, Journal of Virology, 71, 3293-3298, (1997), A helper dependent system for adenovirus vector production helps define a lower limit for
20 efficient DNA packaging). In this invention, PAV genomic length was 34.8 kb, into which was inserted without any other deletion an expression cassette of 2.38 kb. The resulting genomic DNA length of the recombinant porcine adenovirus of this invention was 106.8%, and therefore exceeded the putative maximum limit for construction of a stable recombinant. The recombinant virus
25 was plaque purified three times and passaged stably in primary pig kidney cells. The recombinant was shown to contain gp55 by Southern blot hybridisation. Expression of gp55 was demonstrated by infecting primary PK cell line grown on glass cover slips with the recombinant porcine adenovirus. After 24 hours, immunofluorescent staining (IF) showed infected cells expressing gp55.

2 Construction of recombinant PAV-G-CSF

An expression cassette comprising of the porcine adenovirus major late promoter, the gene encoding porcine granulocyte-colony stimulating factor (G-CSF) and SV40 polyA was inserted into the *Sma*I site of the right hand end (MU 5 97-99.5) of porcine adenovirus serotype 3 and used to generate in porcine primary kidney cells a recombinant PAV 3. The size of the expression cassette was 1.28 kilobase pairs. No deletion of the genomic PAV 3 was made. The recombinant virus was plaque purified two times and passaged stably in primary pig kidney cells. The recombinant was shown to contain G-CSF by Southern blot hybridisation and polymerase chain reaction (PCR). Expression of G-CSF was demonstrated by infecting primary kidney cells with the recombinant PAV-G-CSF. Tissue culture supernatants from the infected primary kidney cells were then electrophoresed in SDS-PAGE gels and transferred to filters. Infected cells expressing G-CSF were detected in a Western blot using a rabbit polyclonal 10 antiserum against porcine G-CSF expressed by purified recombinant *E coli*.

3 Construction of recombinant PAV-gp55T/GM-CSF

An expression cassette consisting of the porcine adenovirus major late promoter, a truncated form of the classical swine fever virus gene gp55 fused in frame to the gene encoding either the full length or the mature form of porcine 20 granulocyte/macrophage-colony stimulating factor (GM-CSF) and SV40 polyA was inserted into the *Sma*I site of the right hand end (MU 97-99.5) of porcine adenovirus serotype 3 and used to generate in porcine primary kidney cells a recombinant PAV 3. The size of the expression cassette was 2.1 kilobase pairs. No deletion of the genomic PAV 3 was made. The recombinant virus was 25 plaque purified two times and shown to contain gp55 and GM-CSF by PCR.

4 Construction of recombinant PAV-gp55/E3

An expression cassette consisting of the porcine adenovirus major late promoter, the classical swine fever virus gene gp55 and SV40 polyA was inserted between the *Bsr*GI/*Sna*BI sites in the E3 region (MU 81-84) of porcine 30 adenovirus serotype 3 and used to generate in porcine primary kidney cells a

recombinant PAV 3. The size of the expression cassette was 2.38 kilobase pairs. A 620 base pair deletion of the genomic PAV 3 was made. The recombinant virus was plaque purified two times and shown to contain gp55 by PCR.

5 VACCINATION STRATEGY

1. Vaccination with PAV-gp55

In this experiment 5-6 week old piglets were used to represent immunocompetent pigs. A group of the piglets (#2, 6 and 7) were vaccinated with recombinant PAV-gp55 administered subcutaneously at a dose of 1×10^7 pfu per piglet. A control group of piglets (#3, 8, 11, 12, 13 and 14) were unvaccinated. No clinical signs were observed (no rise in temperature) in the vaccinated group of piglets (Table 1).

Table 1. Temperatures of pigs vaccinated with rPAV::gp55

Pig No.	Temperatures post vaccination with rPAV::gp55 CSFV (°C)							
	Day 0	1	2	3	6	9	10	13
2	39.7	39.2	39.4	39.8	39.6	39.8	39.6	39.2
3 (control)	39.5	39.2	39.4	39.0	38.8	39.3	39.0	39.7
6	39.7	39.1	39.1	39.0	39.1	39.8	39.1	39.8
7	39.4	39.8	39.8	39.4	39.9	38.9	39.6	39.7
8 (control)	39.6	39.5	39.4	39.0	40.5	39.4	39.1	39.7

Five weeks after vaccination with the recombinant PAV-gp55 both groups of pigs were challenged with a lethal dose ($1 \times 10^{3.5}$ TCID₅₀) of virulent Hog Cholera virus (Classical swine fever virus) applied subcutaneously.

The temperatures of the pigs were monitored and the results tabulated in Table 2 and graphically represented in Figure 7.

Table 2 Temperatures post challenge with CSFV (°C)

	Pig No.	Day									
		1	2	3	4	5	6	7	8	9	10
5	14	15	16								
	2	39.6	39.9	40.1	40.3	40.1	39.2	39.7	39.5	39.5	3 9
	7	39.4	39.1	39.5	39.1	40.0	39.4	38.9			
10	3	39.6	40.4	39.6	40.0	40.7	40.7	41.9	40.7	40.9	42.0+
	6	39.5	39.5	40.0	40.0	39.6	40.5	39.9	39.2	39.2	3 8
	8	39.3	38.8	38.9	39.6	39.1	38.9				
15	7	39.8	39.9	40.4	40.6	40.3	39.7	39.7	39.7	39.5	3 9
	3	39.1	39.3	39.6	40.6	39.7	39.8	39.7			
	8	39.9	40.6	40.5	40.3	40.0	41.4	39.8	41.0	40.6	39.0+
	11	39.6	39.9	40.0	40.3	40.7	40.5	40.0	41.8	41.5	41.3+
	12	39.8	39.9	40.9	41.0	41.2	40.6	40.1	41.0	41.7	40.3+
20	13	39.7	40.0	41.2	41.5	41.6	41.0	39.7+			
	14	39.3	40.0	39.6	39.8	40.3	40.7	41.2	40.8	40.2	41.7+

The results show that by day 5 the control group had elevated temperatures (greater than 40.5°C) and showed clinical signs of disease. The vaccinated group showed no clinical signs of disease. Pigs from the control group were dead or euthanased by day 9. The vaccinated group were euthanased at day

- 5 16. At post mortem all control pigs showed severe clinical disease, the vaccinated pigs showed no clinical signs of disease.

The results indicate that the pigs vaccinated subcutaneously with the recombinant PAV-gp55 survived challenge with classical swine fever virus at a lethal dose.

- 10 Sera were collected from both groups of pigs and tested for the presence of antibodies to PAV by ELISA. These tests showed the presence of pre-existing antibodies to PAV before vaccination. The level of these antibodies increased following vaccination with the recombinant PAV-gp55 to peak between days 28 and 36 post vaccination. These results are tabulated in Figure
15 8.

- Sera were collected from the vaccinated group of pigs pre and post challenge with CSFV and tested in the presence of neutralising antibodies to CSFV. Sera were tested at days 0 and 28 after vaccination with recombinant PAV-gp55 (pre challenge) and then again at day 16 post challenge (day 52 after
20 vaccination). The results in Figure 9 show no neutralising antibodies detected at day 0, low levels of neutralising antibodies at day 28 and high levels at day 52. These results show that the recombinant PAV-gp55 can protect pigs from lethal challenge with classical swine fever virus in the presence of pre-existing antibodies to PAV.

25 2. Vaccination with PAV-G-CSF

- In this experiment 5-6 week old piglets were used to represent immunocompetent pigs. A group of pigs (n=4) were vaccinated with recombinant PAV-G-CSF administered subcutaneously at a dose of 1×10^7 pfu per piglet. A second group (n=4) were vaccinated with PAV wild type (wt)
30 administered subcutaneously at a dose of 1×10^7 pfu per piglet. A control group (n=4) were unvaccinated. Pigs were bled at 8 hour intervals for a period of 104 hours post vaccination. Complete blood counts were determined and the

mean white blood cell (WBC) counts for each group monitored. These results are graphically represented in Figure 10 and the percentage change in mean WBC counts graphically represented in Figure 11.

Pigs vaccinated with either PAV wt or PAV-G-CSF showed clinical signs of disease with mild diarrhoea 24-72 hours post vaccination. Both groups of pigs were completely recovered by 80-96 hours post-vaccination. Control pigs showed no clinical signs of disease.

Complete blood screening results show that the mean WBC counts for control pigs increased over the duration of the experiment.

PAV wt vaccinated pigs also show an increase in WBC counts, with a depression in WBC counts between 48-80 hours post-vaccination and recovery from 80-96 hours onwards.

Pigs vaccinated with the recombinant PAV-G-CSF show a significant depression in WBC counts over the duration of the experiment. A statistical analysis of these results is tabulated in Table 3. The analysis shows that differences between the mean WBC counts (controls and PAV-G-CSF; PAV wt and PAV-G-CSF) were significant indicating that the recombinant PAV-G-CSF altered the proportions of cells involved with immunity.

Table 3. Results of t-tests between mean WBC counts of groups of pigs vaccinated with either PAV wild type (wt), PAV recombinant expressing G-CSF (PAV-G-CSF) or unvaccinated controls.

	Pre vacc 0 hr	8-24 hr ^d	32-48 hr	56-72 hr	80-104 hr
Control vs PAV - G - CSF ^a	p> 0.2 ^b	p> 0.2	p> 0.2	p> 0.2	p< 0.005
Control vs PAV wt	p> 0.1	p> 0.01 ^c	p> 0.02	p> 0.2	p< 0.05
PAV-G-CSF vs PAV wt	p> 0.2	p< 0.05	p< 0.05	p< 0.05	p< 0.001

a: null hypothesis; there is no difference between the mean WBC counts.

b: p>0.05, insufficient to reject the null hypotheses at the 95% confidence level, conclude that there is no difference between mean leucocyte levels.

c: p<0.05, null hypothesis rejected at 95% confidence level, conclude that there is a difference between the mean leucocyte levels.

d: 4 pigs in each group were bled at 8 hour intervals.

Differential WBC counts were also determined and monitored for each group. The percentage change in mean monocyte cell populations graphically represented in Figure 12 and the percentage change in mean lymphocyte cell populations graphically represented in Figure 13. Figure 12 shows that monocyte cell populations increased rapidly in pigs following vaccination with PAV wt, but were suppressed by vaccination with the recombinant PAV-G-CSF. This effect was due to the expression of G-CSF by the recombinant. A statistical analysis of these results is tabulated in Table 4. The analysis shows that there was a significant difference between the PAV wt and PAV-G-CSF from 32 to 96 hours post vaccination. Figure 13 shows that there were shifts in lymphocyte cell population numbers following vaccination with the recombinant PAV-G-CSF. Unvaccinated controls show stable lymphocyte cell numbers over the duration of the experiment, whereas pigs vaccinated with PAV wt show a significant increase in lymphocyte cell population as a response to infection. Pigs vaccinated with the recombinant PAV-G-CSF show a decline in lymphocyte cell population. A statistical analysis of these results is tabulated in Table 5. The analysis shows that there was a significant difference between PAV wt and the recombinant PAV-G-CSF between 8 and 96 hours post vaccination. The different responses in lymphocyte cell proliferation following vaccination with recombinant PAV-G-CSF and PAV wt were due to the expression of G-CSF by the recombinant. These results show that vaccination with recombinant PAV-G-CSF produces a shift in sub-populations of cells involved in immunity.

Table 4. Results of t-tests between mean monocyte cell populations following vaccination of pigs with either recombinant PAV-G-CSF, wild type PAV (PAV wt) or unvaccinated controls.

	pre vacc	8-24hr ^d	32-48hr	56-72hr	80-96hr	104hr
controls vs PAV-G-CSF ^a	p>0.1 ^b	p>0.2	p>0.2	p>0.2	p>0.2	p>0.2
controls vs PAV wt	p>0.2	p<0.002 ^c	p>0.2	p<0.001 ^c	p>0.2	p>0.2
PAV wt vs PAV-G-CSF	p>0.2	p<0.001 ^c	p>0.2	p>0.2	p>0.1	p>0.05

a: null hypothesis; there is no difference between the mean monocyte cell counts.

b: p>0.1, insufficient to reject the null hypothesis at the 90% confidence level, conclude that there is no difference between mean monocyte cell levels.

c: p<0.05, null hypothesis rejected at 95% confidence level, conclude that there is a difference between the mean monocyte cell levels

d: 4 pigs in each group were bled at 8 hour intervals

Table 5. Results of t-tests between mean lymphocyte cell populations following vaccination of pigs with either recombinant PAV-G-CSF, wild type PAV (PAV wt) or unvaccinated controls.

	pre vacc	8-24hr ^d	32-48hr	56-72hr	80-96hr	104hr
5 controls vs PAV-G-CSF ^a	p>0.2	p>0.05 ^b	p>0.2	p>0.2	p>0.2	p>0.2
controls vs PAV wt	p>0.2	p>0.2	p<0.01 ^c	p<0.001 ^c	p<0.001 ^c	p>0.2
PAV wt vs PAV-G-CSF	p>0.2	p<0.05 ^c	p<0.002 ^c	p<0.005 ^c	p<0.001 ^c	p>0.05

- 10 a: null hypothesis; there is no difference between the mean lymphocyte cell counts.
 b: p>0.05, insufficient to reject the null hypothesis at the 95% confidence level, conclude that there is no difference between mean lymphocyte cell levels.
 c: p<0.05, null hypothesis rejected at 95% confidence level, conclude that there is a difference between the mean lymphocyte cell levels.
 15 d: 4 pigs in each group were bled at 8 hour intervals

Figure 14 graphically represents changes in the proliferation of T cells of each group following stimulation with Concanavalin A (Con A). These results confirm that there was a significant proliferation of T-cells following vaccination with PAV wt at day 2 post vaccination, whereas vaccination with the recombinant PAV-G-CSF resulted in a suppression of T-cell proliferation by day 3.

The results of vaccination with a recombinant PAV expressing porcine G-CSF shows that G-CSF has a significant effect on the cells involved with immune responses.

- 25 It will be appreciated that whilst this document establishes the metes and bounds of this invention, all embodiments falling within its scope for example with regard to heterologous genes, insertion sites, types of promoter and serotype have not necessarily been specifically exemplified although it is intended that they should fall within the scope of protection afforded this
 30 invention.

Figure 2

Total sequence of the PAV Major Late Promoter cassette including the added nucleotides 5' (upstream) of the USF.

Nucleotide base count: 76 A 143 C 187 G 96 T Total 502 bp

```

1   GGTGCCGCGG TCGTCGGCGT AGAGGATGAG GGCCCACTCG GAGATGAAGG CACGCGCCCA
61  GGCAGAGGACG AAGCTGGCGA CCTGCGAGGG GTAGCGGTCG TTGGGCACTA ATGGCGAGGC
121 CTGCTCGAGC GTGTGGAGAC AGAGGTCCTC GTCGTCCGCG TCCAGGAAGT GGATTGGTCG
181 CCAAGTGGTAG TCCACGTGAC CGGCTTGCGG GTCGGGGGGT ATAAAAGGCG CGGGCCGGGG
241 TCGTGCGCCG TCAGTTGCTT CGCAGGCCTC GTCACCGGAG TCCGCGTCTC CGGCGTCTCG
301 CGCTGCGGCT GCATCTGTGG TCCCGGAGTC TTCAGGTCCT TGTGAGGAG GTACTCCTGA
361 TCGCTGTCCC AGTACTTGGC GTGTGGGAAG CCGTCCTGAT CGCGATCCTC CTGCTGTTGC
421 AGCGCTTCGG CAAACACGCG CACCTGCTCT TCGGACCCGG CGAAGCGTTC GACGAAGGCG
481 TCTAGCCAGC AACAGTCGCA AG

```

The Upstream Stimulatory Factor (USF) and TATA motif are in **bold**. The complete leader sequence is italicised with the cap site and splice sites between the individual leaders indicated by double underlining or single underlining respectively.

Figure 3

Individual sequences of the Promoter cassette components:

I. The 5' (upstream) sequence included in the long cassette.

```

1      GGTGCCGCGG TCGTCGGCGT AGAGGATGAG GGCCCGAGTCG GAGATGAAGG CACGCGCCCA
61     GGCGAGGACG AAGCTGGCGA CCTGCGAGGG GTAGCGGTCG TTGGGCACTA ATGGCGAGGC
121    CTGCTCGAGC GTGTGGAGAC AGAGGTCTCT GTCGTCCGCG TCCAGGAAGT GGATTGGTCG
181    CCAGTGGTAG

```

II. Sequence including the USF, TATA motif and sequence to the cap site.

```

1      CCACGTGACC GGCTTGCGGG TCGGGGGGTA TAAAAGGCGC GGGCCGGGGT GCGTGGCCGT
61     C

```

III. First leader sequence.

```

1      AGTTGCTTCG CAGGCCTCGT CACCGGAGTC CGCGTCTCCG GCGTCTCGCG CTGCGGCTGC
61     ATCTGTGGTC CCGGAGTCTT CAG

```

IV. Second leader sequence.

```

1      GTCCTTGTTG AGGAGGTACT CCTGATCGCT GTCCCAGTAC TTGGCGTGTG GGAAGCCGTC
61     CTGATCG

```

V. Third leader sequence.

```

1      CGATCCTCCT GCTGTTGCAG CGCTTCGGCA AACACGCGCA CCTGCTCTTC GGACCCGGCG
61     AAGCGTTCGA CGAAGGCGTC TAGCCAGCAA CAGTCGCAAG

```

Figure 4

Sequence of the right hand end of the PAV genome this area being a proposed site for insertion of expression cassettes.

Nucleotide base count 183 A 255 C 306 G 204 T Total 948 bases

```

1 CATCATCAAT AATATACCGC ACACCTTTTAT TGCCCCCTTTT GTGGCGTGGT GATTGGCGGA
61 GAGGCTTGGG GCGGGCGGGC GGTGATTGGT GGAGAGGGGT GTGACGTAGC GTGGGAACGT
121 GACGTCGCGT GGGAAAATAA CGTGGCGTGG GAACGGTCAA AGTCCGAGGG CCGGGGTCAA
181 AGTCCGCGT CCGGGGCGG AGCCGGCTGG CGGGAATTCC CCGGACTTTC TGGGCGGGTA
                               EcoRI SmaI
241 ATCGTTAAG CGGAGGCGGG GGAATTCGA TCGGACGATG TGGTACTGAT TAACCGACCG
      HpaI EcoRI
301 CAGGCGTGT CACATCCGCT GTGGGTATAT CACCGGCGCT CGCGGTGTTT GCTCACACTC
361 GTCTCGGCGC TGTCACAGAG AGAGACACTG AGAGCGAGAC GAGGAGAAAC CGAAAGCGGG
421 GCAGGAGGAG TCACCGGGCC ATCTTCCCAT CAGAGCCCTC TCATGGCCCA CGACCGACTG
481 CTGCTGGCCG CGGTGGCTGA CTGTTGCTCG CCGTGCTCTA TCTGTACTTC GCCTACCTCG
541 CGTGGCAGGA TCGGGACACT CTTCACACTC AGGAGGCCGC CTCTCCTCGC TTCTTCATCG
601 GGTCCAACCA CCAGCCCTGG TGCCCGGATT TTGATTGGCA GGAGCAGGAC GAGCACACTC
661 ACTAGACGTT TAGAAAAAAG ACACACATTG GAACTCATAT ATGTCTGCGG GACCGCATCA
721 GCAGCCCGGT CTGCTGTTGG CTGCGGGTGA GAGGCCTCCG GTAATTCATC AGAACCGCAT
                               StuI
781 TCATCTGCGC CACGTCCCGA CATATGGTGC TGACGTCAGA ACAGCCCAGC GTGATCCTTT
                               SacIII
841 TAATGTGCTA GTCTACGTGC CCACTGGGTT TGCTGTGTTT GTGCCGACTG AGCGAGATTT
901 TCAGAGGAGG GATCTGGTCC GTTTCAGAC CTGCTGCTTC CGGCATCA

```

The Inverted Terminal Repeat (ITR) is shown in bold. Enzyme sites of interest are underlined with the enzyme name below. Putative TATA for E4 region is also shown.

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A recombinant porcine adenovirus capable of expressing DNA of interest, said DNA of interest being stably integrated into an appropriate site of said recombinant porcine adenovirus genome.
2. A recombinant vector including a recombinant porcine adenovirus stably incorporating, and capable of expressing DNA of interest.
3. A recombinant vector as claimed in claim 2 wherein said recombinant porcine adenovirus is capable of expression of at least one heterologous nucleotide sequence.
4. A recombinant vector as claimed in claims 2 or 3 wherein said recombinant porcine adenovirus includes a live porcine adenovirus having virion structural proteins unchanged from those in a native porcine adenovirus from which said recombinant porcine adenovirus is derived.
5. A recombinant vector as claimed in claims 3 or 4 wherein said at least one heterologous nucleotide sequence is capable of expression as an antigenic polypeptide.
6. A recombinant vector as claimed in claims 3 or 4 wherein said at least one heterologous nucleotide sequence is capable of expression as an immuno-potentiating molecule.
7. A recombinant vector as claimed in claim 5 wherein said heterologous nucleotide sequence encodes antigenic determinants of infectious agents causing intestinal diseases in pigs.

8. A recombinant vector as claimed in claim 5 wherein said heterologous nucleotide sequence encodes antigenic determinants of infectious agents causing respiratory diseases in pigs.
9. A recombinant vector as claimed in claim 5 where said heterologous sequence encodes an antigenic determinant of pseudorabies virus (Aujeszky's disease virus).
10. A recombinant vector as claimed in claim 9 where heterologous sequence encodes an antigenic determinant of glycoprotein D of pseudorabies virus.
11. A recombinant vector as claimed in claim 5 where said heretologous sequence encodes an antigenic determinant of porcine respiratory and reproductive syndrome virus (PRRSV).
12. A recombinant vector as claimed in claim 5 wherein said heterologous nucleotide sequence encodes an antigenic determinant of Hog cholera virus.
13. A recombinant vector as claimed in claim 5 wherein said heterologous nucleotide sequence encodes an antigenic determinant of porcine parvovirus.
14. A recombinant vector as claimed in claim 5 wherein said heterologous nucleotide sequence encodes an antigenic determinant of porcine coronavirus.
15. A recombinant vector as claimed in claim 5 wherein said heterologous nucleotide sequence encodes an antigenic determinant of porcine rotavirus.
16. A recombinant vector as claimed in claim 5 wherein said heterologous nucleotide sequence encodes an antigenic determinant of porcine parainfluenza virus.

17. A recombinant vector as claimed in claim 5 wherein said heterologous nucleotide sequence encodes an antigenic determinant of *Mycoplasma hyopneumoniae*.
18. A recombinant vector as claimed in claim 6 wherein said heterologous nucleotide sequence encodes FLT-3 ligand.
19. A recombinant vector as claimed in claim 6 wherein said heterologous nucleotide sequence encodes interleukin 3 (IL-3).
20. A recombinant vector as claimed in claim 6 wherein said heterologous nucleotide sequence encodes porcine interleukin 4 (IL4).
21. A recombinant vector as claimed in claim 6 wherein said heterologous nucleotide sequence encodes gamma interferon (γ IFN).
22. A recombinant vector as claimed in claim 6 wherein said heterologous nucleotide sequence encodes porcine granulocyte macrophage colony stimulating factor (GM-CSF).
23. A recombinant vector as claimed in claim 6 wherein said heterologous nucleotide sequence encodes porcine granulocyte colony stimulating factor (G-CSF).
24. A recombinant vector as claimed in claims 3 or 4 wherein said heterologous nucleotide sequence encodes an antigenic polypeptide and an immuno-potentiating molecule.
25. A recombinant vector as claimed in any one of claims 2 to 24 wherein said recombinant porcine adenovirus is selected from the group consisting of serotypes 3 and 4.

26. A recombinant vector as claimed in any one of claims 2 to 25 wherein DNA of interest is stably integrated into the non-essential regions of the porcine adenovirus genome.
27. A recombinant vector as claimed in any one of claims 2 to 26 wherein DNA of interest is stably integrated into the right hand end of the genome.
28. A recombinant vector as claimed in claim 27 wherein DNA of interest is stably integrated into the right hand end of the genome at map units 97 to 99.5.
29. A recombinant vector as claimed in any one of claims 2 to 26 wherein DNA of interest is stably integrated into the E3 region of the genome.
30. A recombinant vector as claimed in claim 29 wherein DNA of interest is stably integrated into the E3 region of the genome at map units 81-84.
31. A method of producing a recombinant porcine adenovirus vector for use as a vaccine including inserting into a non-essential region of an porcine adenovirus genome, at least one heterologous nucleotide sequence in association with an effective promoter sequence.
32. A method as claimed in claim 31 wherein prior to insertion of said heterologous nucleotide sequence, a restriction enzyme site is inserted into said non-essential region of said porcine adenovirus genome.
33. A recombinant vaccine for generating and/or optimising antibodies or cell mediated immunity so as to provide or enhance protection against infection by an infectious organism in pigs, said vaccine including at least one recombinant porcine adenovirus vector stably incorporating, and capable of expression of at least one heterologous nucleotide sequence, and suitable carriers and/or excipients.

34. A recombinant vaccine as claimed in claim 33 wherein the said at least one heterologous nucleotide sequence is capable of expression as an antigenic polypeptide.

35. A recombinant vaccine as claimed in claim 33 wherein said at least one heterologous nucleotide sequence is capable of expression as an immuno-potentiating molecule.

36. A recombinant vaccine as claimed in claim 33 wherein said heterologous nucleotide sequence encodes an antigenic polypeptide and an immuno-potentiating molecule.

37. A recombinant vaccine as claimed in any one of claims 33 to 36 wherein said carriers and/or excipients are selected such that said vaccine is deliverable in the form of an aerosol spray, an enteric coated dosage unit or an inoculum.

38. A method of producing a recombinant vaccine as claimed in any one of claims 33 to 36 including admixing at least one recombinant porcine adenovirus vector stably incorporating, and capable of expression of at least one heterologous nucleotide sequence together with suitable carriers and/or excipients.

39. A method of vaccination of pigs against disease including administering to said pigs a first recombinant porcine adenovirus vector stably incorporating, and capable of expression of at least one heterologous nucleotide sequence encoding an antigenic determinant of said disease against which vaccination is desired.

40. A method as claimed in claim 39 including administering to said pig a second porcine adenovirus vector including at least one heterologous nucleotide sequence which differs from said at least one heterologous nucleotide sequence incorporated in said first recombinant porcine adenovirus vector.

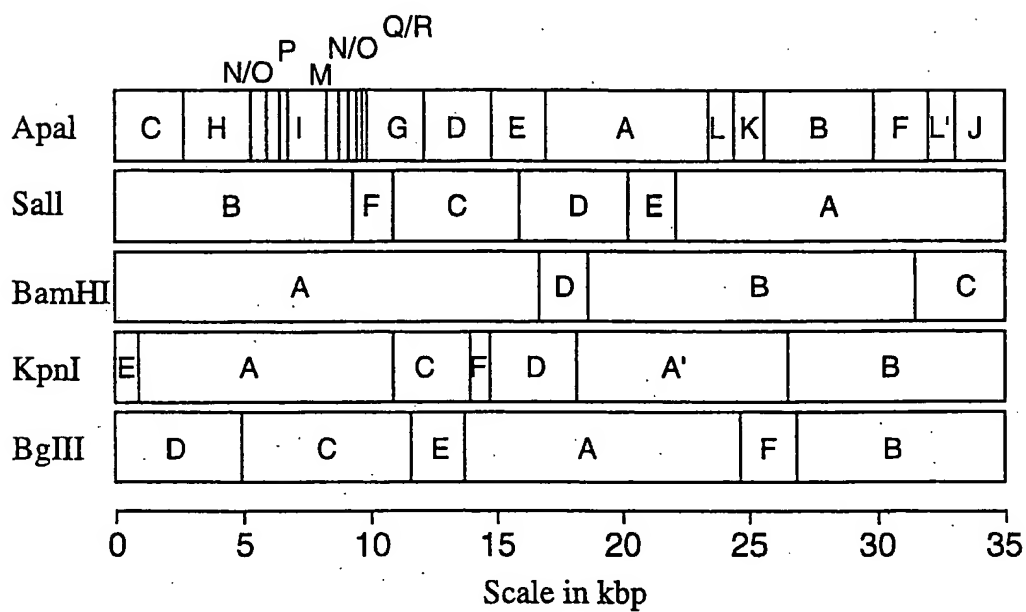
41. A method as claimed in claim 40 wherein said second porcine adenovirus vector comprises a serotype different to that of said first porcine adenovirus vector.

42. A method as claimed in claim 39 wherein said second porcine adenovirus vector incorporates, and is capable of expression of at least one heterologous nucleotide sequence encoding an immuno-potentiating molecule.

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Fig 1.

Restriction enzyme maps of the PAV3 genome



2/14

Fig 2.

Total sequence of the PAV Major Late Promoter cassette including the added nucleotides 5' (upstream) of the USF.

Nucleotide base count: 76 A 143 C 187 G 96 T Total 502 bp

```

1   GGTGCCGCGG TCGTCGGCGT AGAGGATGAG GGCCCAAGTCG GAGATGAAGG CACGCGCCCA
61  GCGGAGGACG AAGCTGGCGA CCTGCGAGGG GTAGCGGTCTG TTGGGCACTA ATGCGGAGGC
121 CTGCTCGAGC GTGTGGAGAC AGAGTCCTCT GTCGTCCGCG TCCAGGAAGT GGATTGGTCTG
181 CCAGTGGTAG TCCACGTGAC CGGCTTGCGG GTCCGGGGGT ATAAAGGCG CGGCGCGGGG
241 TCGTGCGCGG TCAGTTGCTT CGCAGGCCCTC GTCACCGGAG TCCGCGTCTC CGGCGTCTCG
301 CGCTGCGGCT GCATCTGTGG TCCCGGAGTC TTCAGGTCTT TGTTGAGGAG GTACTCCTGA
361 TCGCTGTCCC AGTACTTGGC GTGTGGGAAG CCGTCCTGAT CGCGATCCTC CTGCTGTTGC
421 AGCGCTTCGG CAAACACGCG CACCTGCTCT TCGGACCCGG CGAAGCGTTC GACGAAGGCG
481 TCTAGCCAGC AACAGTCGCA AG

```

The Upstream Stimulatory Factor (USF) and TATA motif are in **bold**. The complete leader sequence is italicised with the cap site and splice sites between the individual leaders indicated by double underlining or single underlining respectively.

3/14

Fig 3.

Individual sequences of the Promoter cassette components:

I. The 5' (upstream) sequence included in the long cassette.

```

1      GGTGCCGCGG TCGTCGGCGT AGAGGATGAG GGCCCAGTCG GAGATGAAGG CACGCGCCCA
61     GGCGAGGACG AAGCTGGCGA CCTGCGAGGG GTAGCGGTCG TTGGGCACTA ATGGCGAGGC
121    CTGCTCGAGC GTGTGGAGAC AGAGGTCCTC GTCGTCCGCG TCCAGGAAGT GGATTGGTCG
181    CCAGTGGTAG

```

II. Sequence including the USF, TATA motif and sequence to the cap site.

```

1      CCACGTGACC GGCTTGCGGG TCGGGGGGTA TAAAAGGCGC GGGCCGGGGT GCGTGGCCGT
61     C

```

III. First leader sequence.

```

1      AGTTGCTTCG CAGGCCTCGT CACCGGAGTC CGCGTCTCCG GCGTCTCGCG CTGCGGCTGC
61     ATCTGTGGTC CCGGAGTCTT CAG

```

IV. Second leader sequence.

```

1      GTCCTTGTTG AGGAGGTACT CCTGATCGCT GTCCCAGTAC TTGGCGTGTG GGAAGCCGTC
61     CTGATCG

```

V. Third leader sequence.

```

1      CGATCCTCCT GCTGTTGCAG CGCTTCGGCA AACACGCGCA CCTGCTCTTC GGACCCGGCG
61     AAGCGTTCGA CGAAGGCGTC TAGCCAGCAA CAGTCGCAAG

```

4/14

Fig 4.

Sequence of the right hand end of the PAV genome this area being a proposed site for insertion of expression cassettes.

Nucleotide base count 183 A 255 C 306 G 204 T Total 948 bases

```

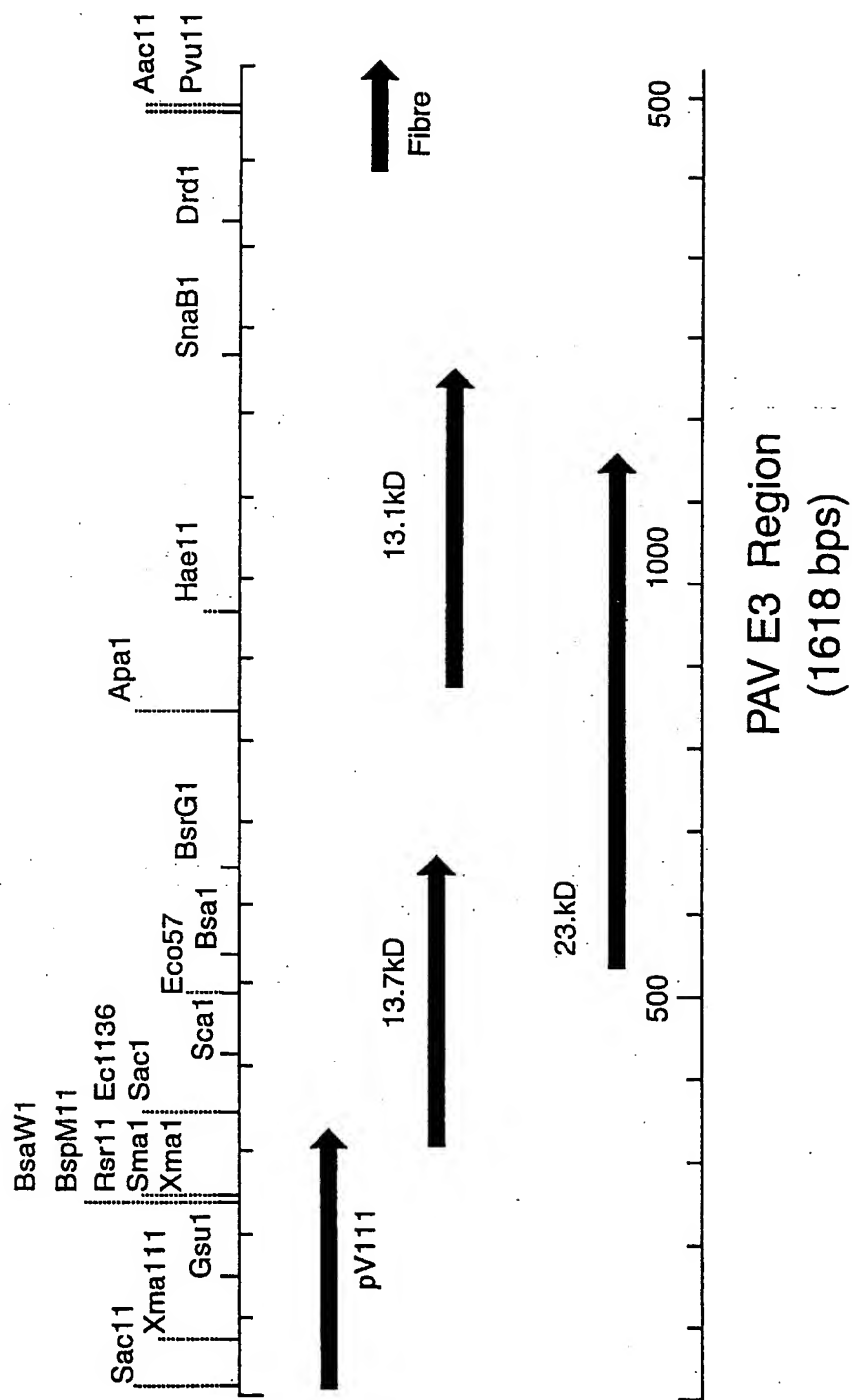
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121 GACGTCGCGT GGGAAATAA CGTGGCGTGG GAACGGTCAA AGTCCGAGGG GCGGGGTCAA
181 AGTCCGCAGT CCGGGGGCGG AGCCGGCTGG CGGGAATTCC CGGACTTTC TGGGCGGGTA
241 ATCGTTAAG HpaI EcoRI SmaI
CGAGGCGGG GGAATTCGA TCGGACGATG TGGTACTGAT TAACCGACCG
301 CAGGCGTGC CACATCCGCT GTGGTATAT CACCGGCGCT CGCGGTGTTT GCTCACACTC
361 GTCTCGGCG TGTCACAGAG AGAGACACTG AGAGCGGAGC GAGGAGAAAC CGAAAGCGGG
421 GCAGGAGGAG TCACCGGGCC ATCTCCCAT CAGAGCCCTC TCATGGCCCA CGACCGACTG
481 CTGCTGGCCG CGGTGGCTGA CTGTGCTCG CCGTGCTCTA TCTGTACTTC GCCTACCTCG
541 CGTGGCAGGA TCGGGACACT CTTACACTC AGAGGCGCGC CTCTCCTCGC TTCTTCATCG
601 GGTCCACCA CCAGCCCTGG TGCCCGGATT TTGATTGGCA GGAGCAGGAC GAGCACACTC
661 ACTAGACGT TAGAAAAAG ACACACATTG GAACTCATAT ATGTCTGCGG GACCGCATCA
721 GCAGCCCGGT CTGCTGTTGG CTGCGGGTGA GAGGCTCCG GTAATTCATC AGAACCGCAT
781 TCATCTGCGC CACGTCCCGA CATATGGTGC TGACGTCAGA ACAGCCCGAG GTGATCCTTT
841 TAATGTGCTA GTCTACGTGC CCACTGGGTT TGCTGTGTTT GTGCCGACTG AGCGAGATTT
901 TCAGAGGAGG GATCTGGTCC GTTCCAGAC CTGCTGCTTC CGGCATCA

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The Inverted Terminal Repeat (ITR) is shown in bold. Enzyme sites of interest are underlined with the enzyme name below. Putative TATA for E4 region is also shown.

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Fig 5.



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Fig 7.

Pig temperatures following CSFV challenge

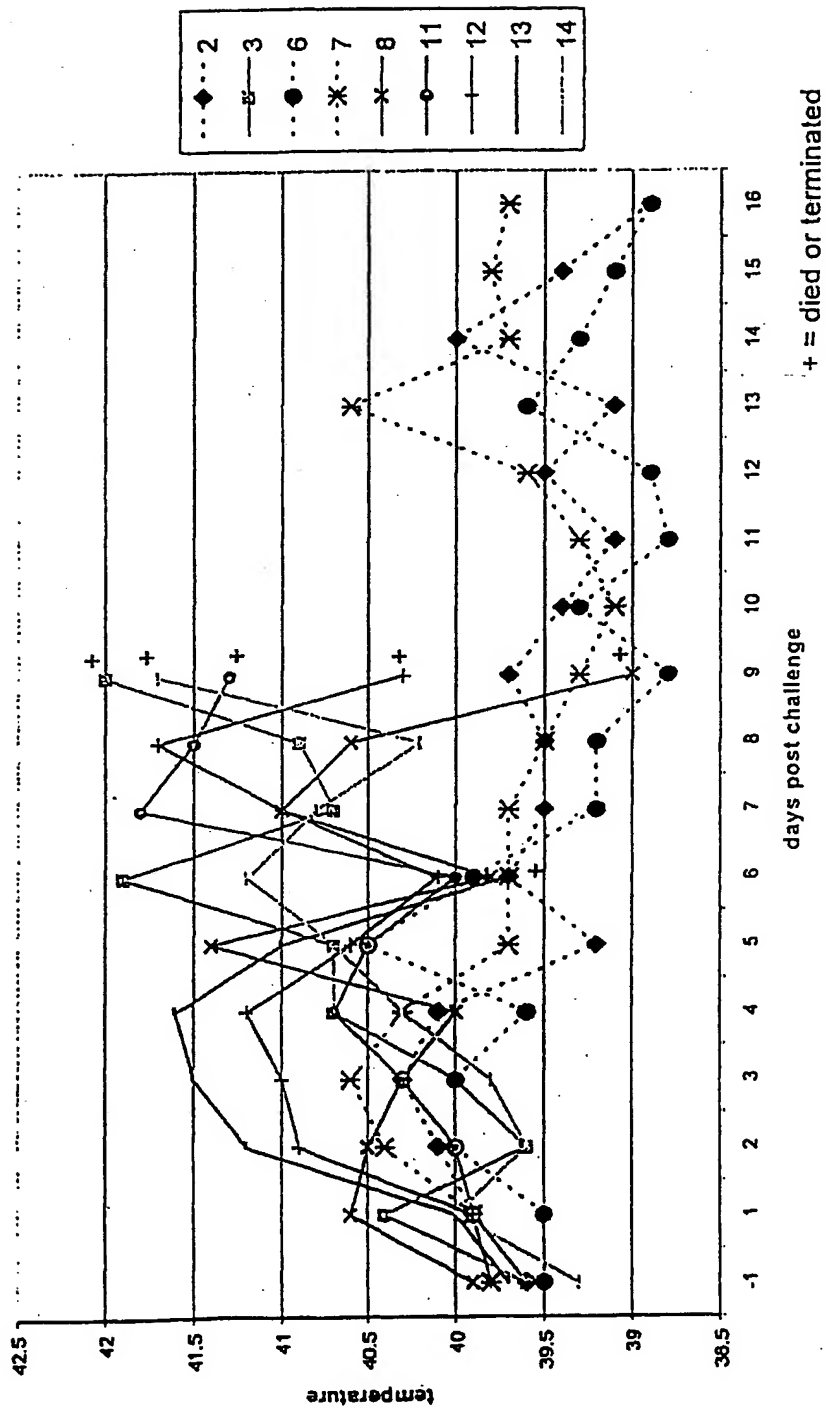


Fig 8.

Anti PAV ELISA titres in pigs following rPAV-gp55 vaccination

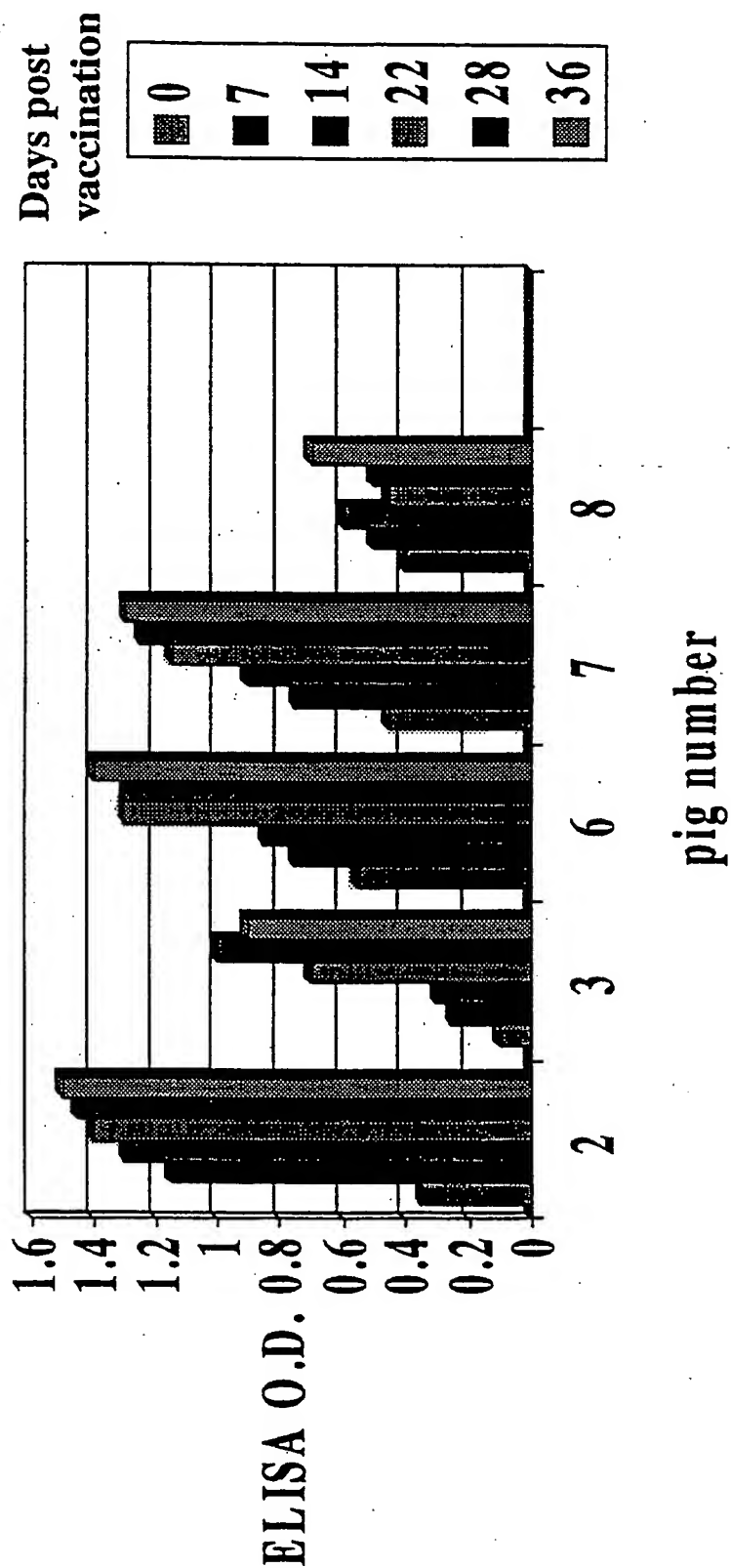
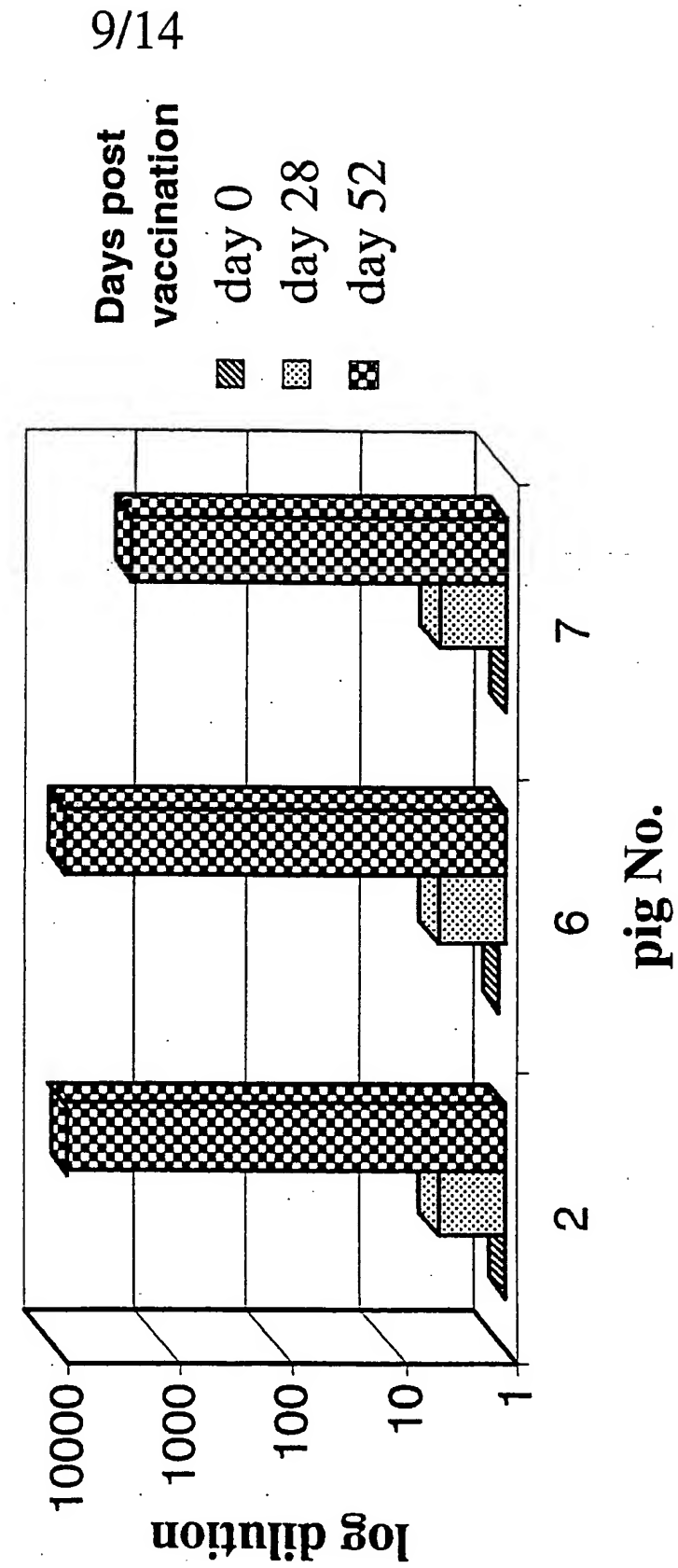


Fig 9.

CSFV SN Ab titres in pig sera



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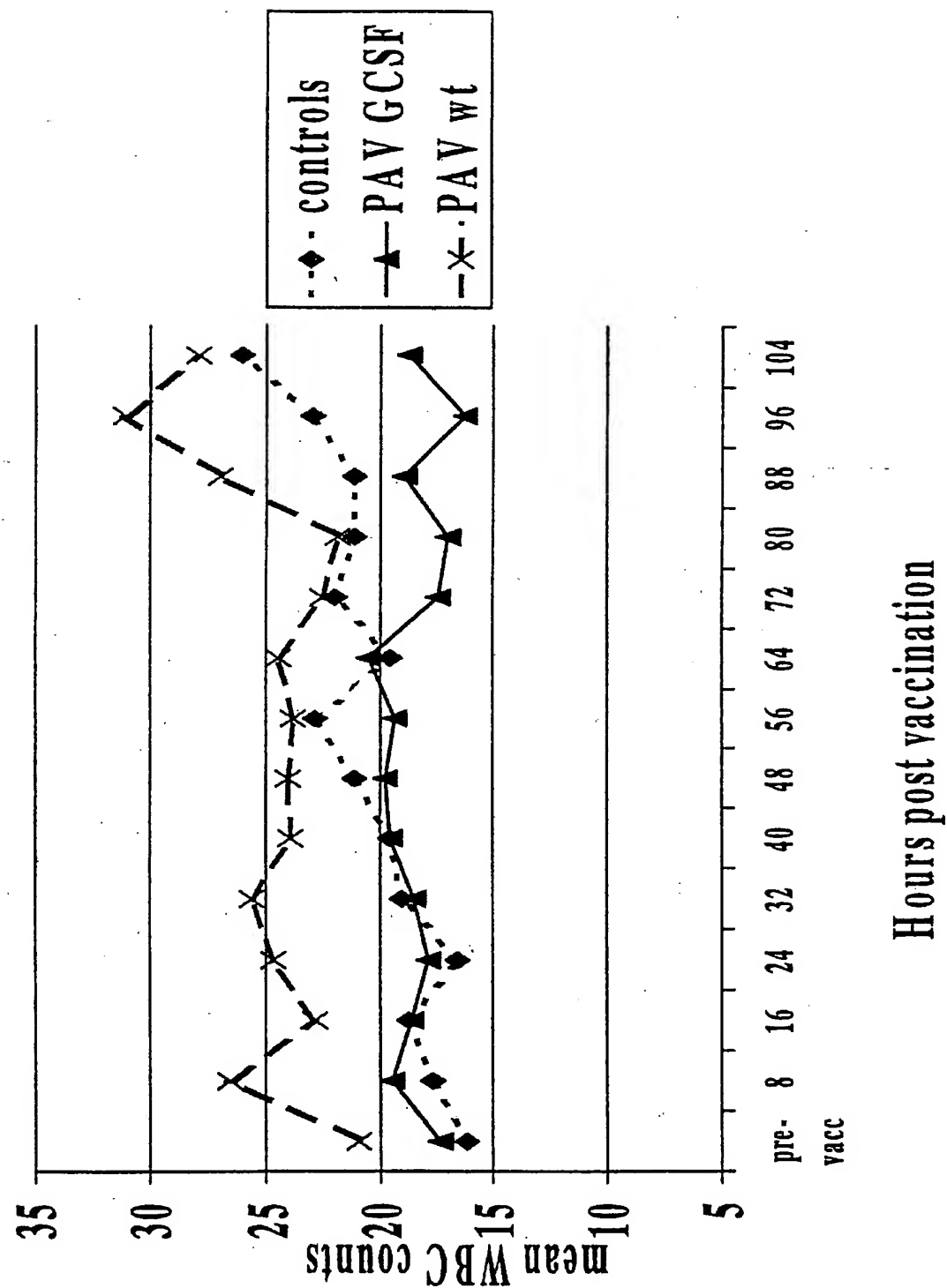
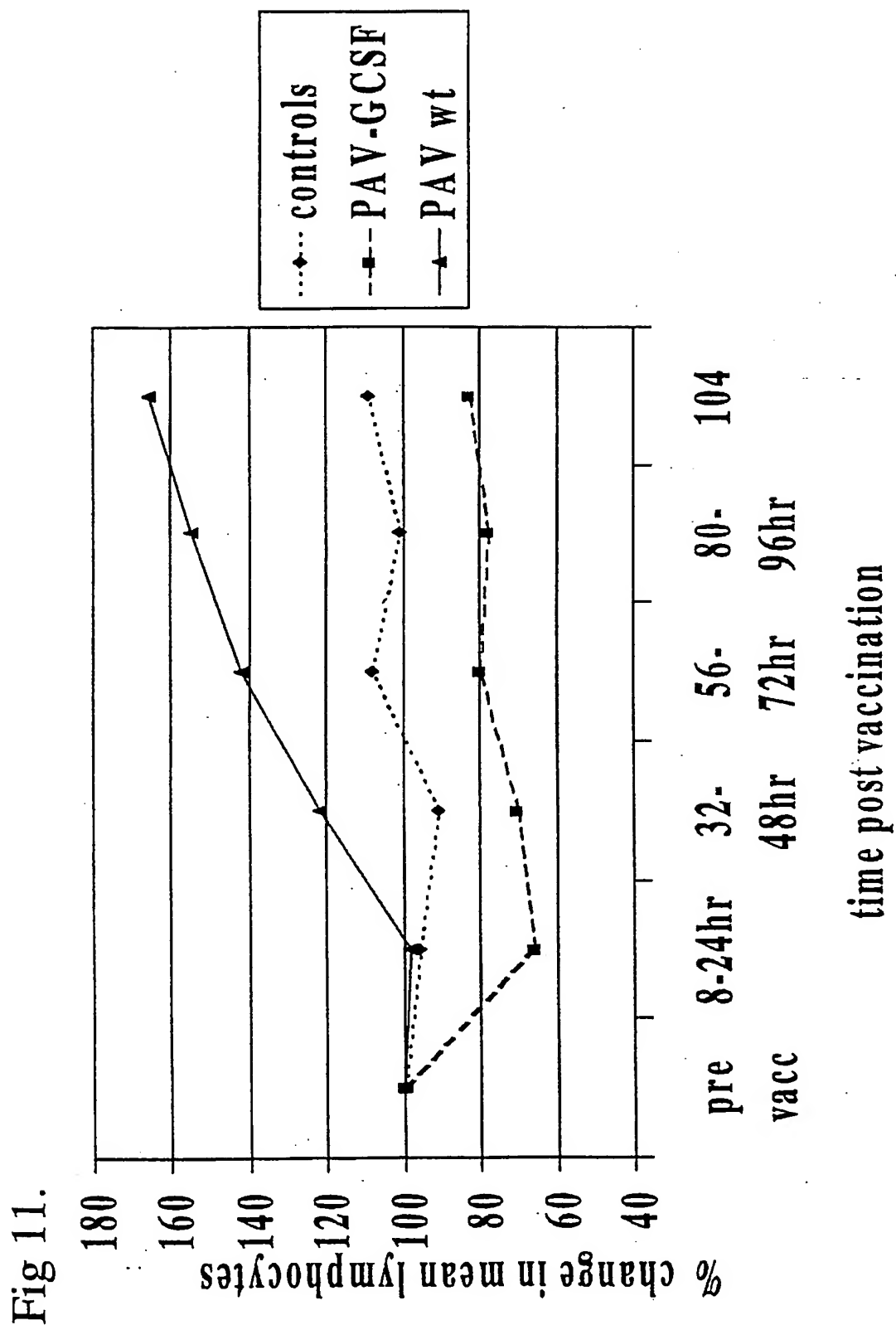
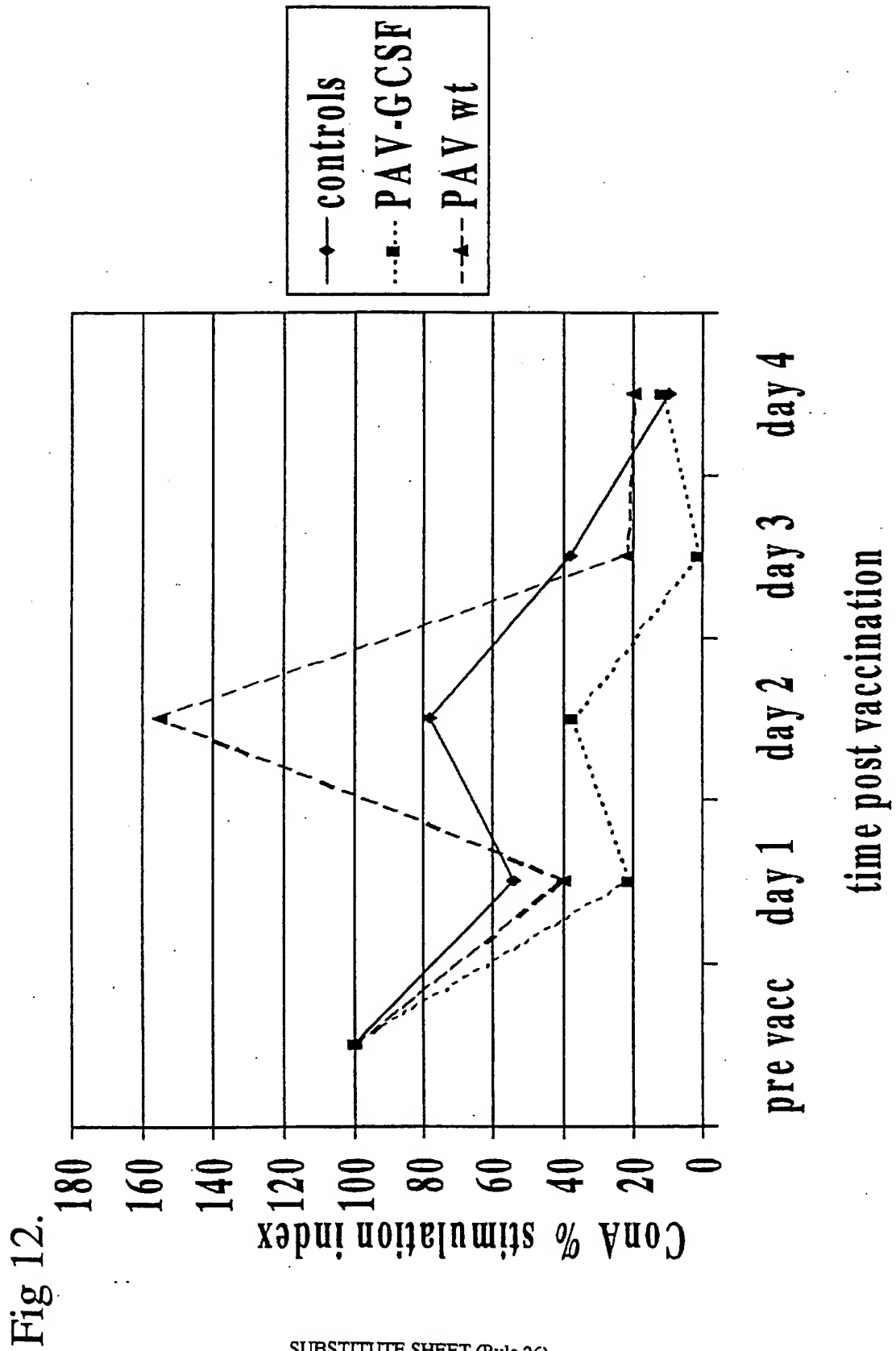


Fig 10.

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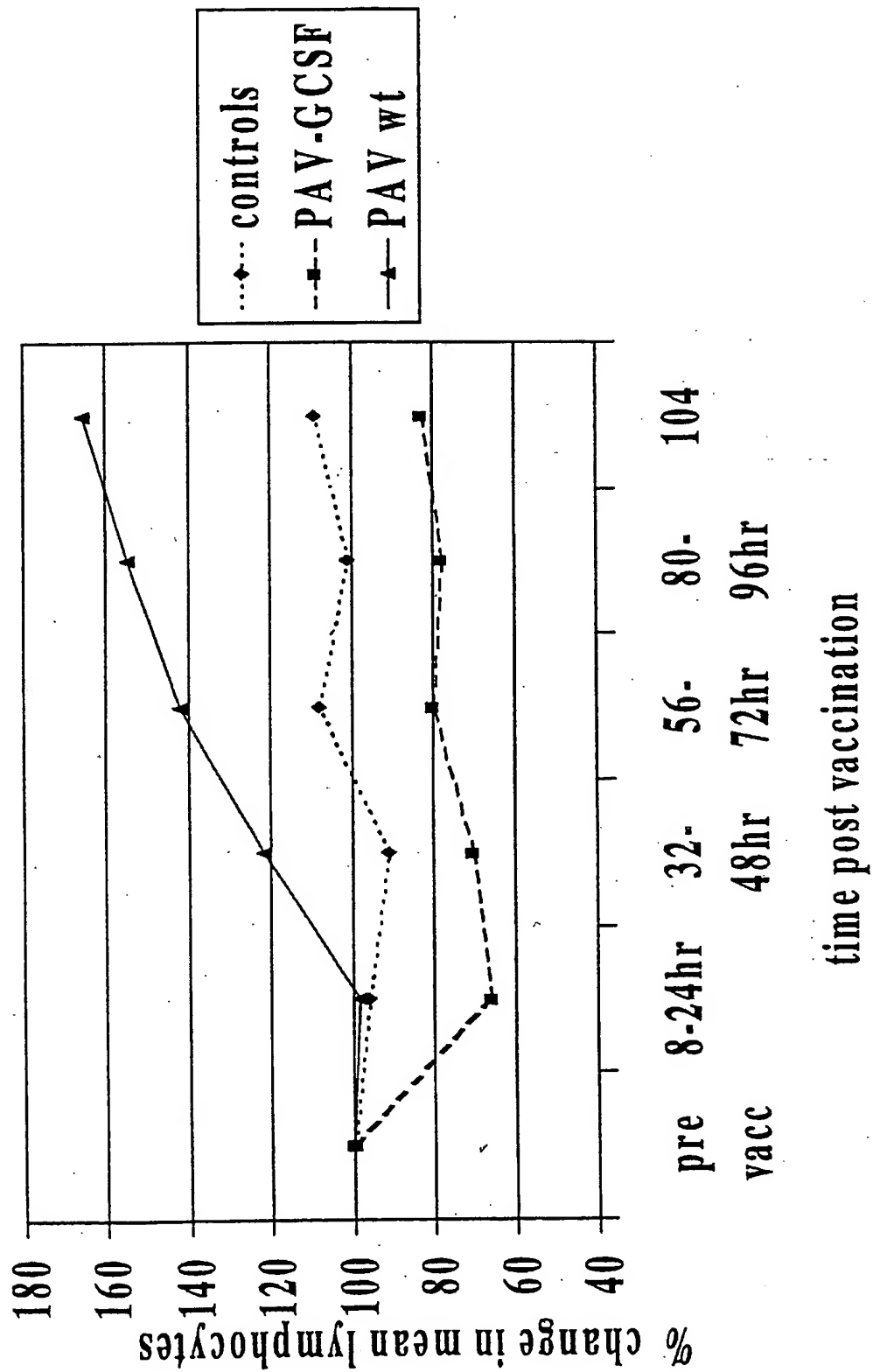
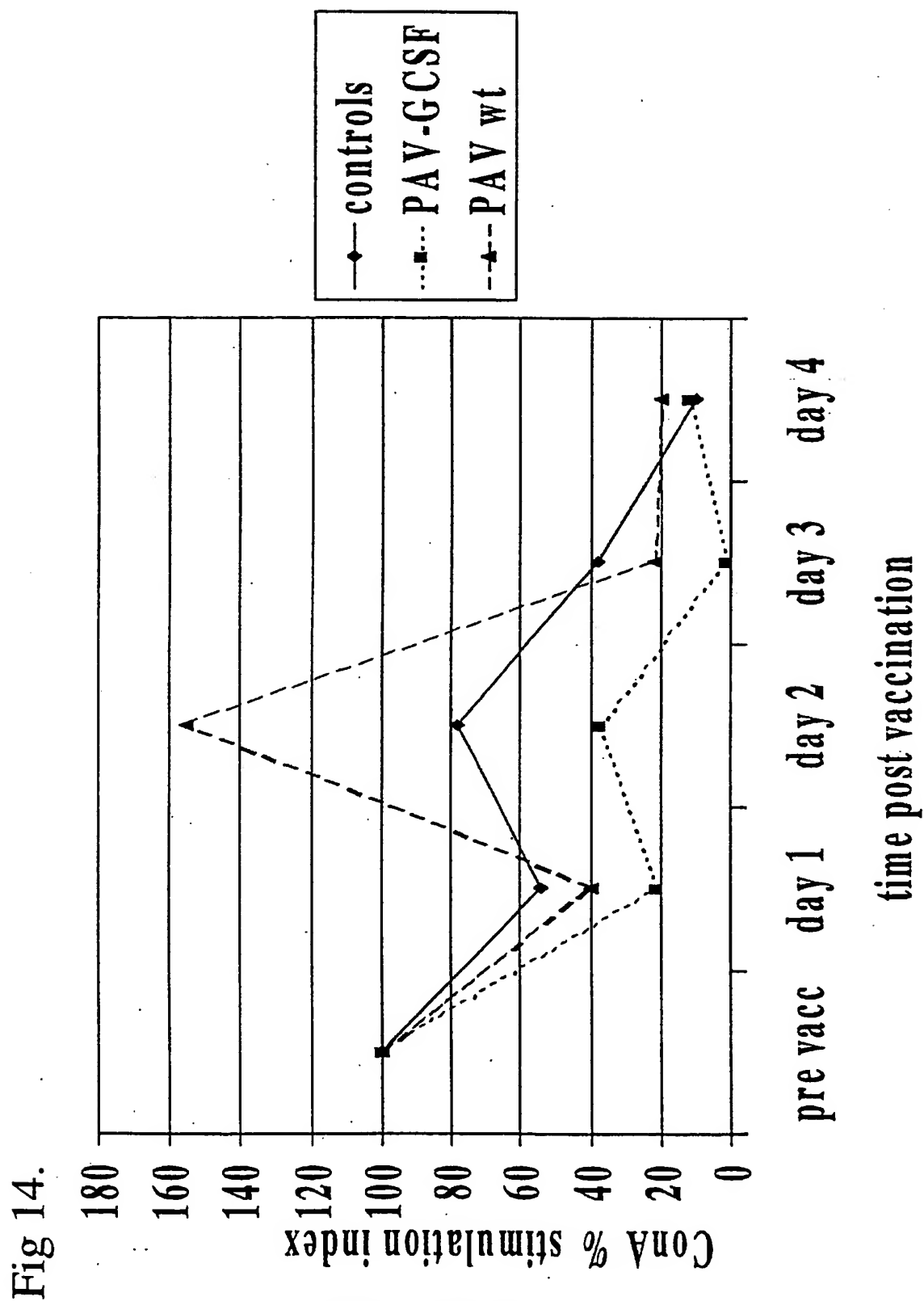


Fig 13.

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU 98/00648

A. CLASSIFICATION OF SUBJECT MATTER

Int Cl⁶: A61K 039/235 ; C12N 15/63, 15/67, 15/86

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
Derwent Database - WPAT ; Chemical Abstracts - Keywords below

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Derwent Database - JAPIO, USPM; Medline - Keywords below

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
WPAT, JAPIO, USPM: Keywords- (swine# or porcine# or hog# or pig#) (20N) adenovir:
Chemical Abstracts, Medline: Keywords- adenovir? (20N) (swine or porcine or hog or pig)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	Derwent Abstract Accession Number: 97-310593 (WO 97/20036 (CYANAMID IBERICA) publ. 5 June 1997) (See examples)	1-4, 25-27, 31-33, 39 <u>5-24, 34-38, 40-42</u>
Y	AU, A, 72646/94 (RHONE POULENC RORER) OPI. 13 February 1995 (see page 2, Examples, Claims 9 and 10)	1-27, 31-42
X Y	Advances in Experimental Medicine and Biology, Vol. 342, 1993, P. Callebaut <u>et al.</u> "Construction of a Recombinant Adenovirus for the Expression of the Glycoprotein S Antigen of Porcine Respiratory Coronaviruses" pp 469- 470 (see entire document)	1-4, 25-27, 31-33, 39 <u>5-24, 34-38, 40-42</u>

☒ Further documents are listed in the
continuation of Box C

☒ See patent family annex

<p>* Special categories of cited documents:</p>	
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Date of the actual completion of the international search
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16 OCT 1998

Name and mailing address of the ISA/AU
AUSTRALIAN PATENT OFFICE
PO BOX 200
WODEN ACT 2606
AUSTRALIA
Facsimile No.: (02) 6285 3929

Authorized officer

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU 98/00648

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	Arch. Virology, Vol. 133, 1993, S.B Kleiboeker <u>et al.</u> , "Genomic Cloning and Restriction Site Mapping of a Porcine Adenovirus Isolate: Demonstration of Genetic Stability in Porcine Adenovirus" pp 357-368 (see entire document) [cited in application]	1-4, 25-27, 31-33, 39 <u>5-24, 34-38,</u> <u>40-42</u>
X Y	J. of Virology, Vol. 66, No. 7, July 1992, M. Gorziglia and A.Z Kapikian, "Expression of the OSU Rotavirus Outer Capsid Protein VP4 by an Adenovirus Recombinant" pp 4407-4412 (see entire document)	1-4, 25-27, 31-33, 39 <u>5-24, 28, 34-</u> <u>38, 40-42</u>
X Y	Virus Research, Vol. 31, 1994, S.B Kleiboeker, "Sequence Analysis of Putative E3. pVIII, and Fiber Genomic Regions of a Porcine Adenovirus" pp 17-25 (see entire document) [cited in application]	1-4, 25-27, 29-33, 39 <u>5-24, 28, 34-</u> <u>38, 40-42</u>
X Y	Virus Research, Vol. 36, 1995, P.S. Reddy <u>et al.</u> , "Sequence Analysis of Putative pVIII, E3 and Fiber Regions of Porcine Adenovirus Type 3" pp 97-106 (see entire document)	1-4, 25-27, 29-33, 39 <u>5-24, 28, 34-</u> <u>38, 40-42</u>
X Y	Virus Research, Vol. 43, 1996, P.S. Reddy <u>et al.</u> , "Porcine Adenoviruses Types 1, 2, and 3 have Short and Simple Early E3 Regions" pp. 99-109 (see entire document)	1-4, 25-27, 29-33, 39 <u>5-24, 28, 34-</u> <u>38, 40-42</u>
X Y	J. of Virology, Vol. 70, No. 6, June 1996, J.M. Torres <u>et al.</u> , "Tropism of human adenovirus Type 5-based vectors in swine and their ability of protect against transmissible gastroenteritis coronavirus" pp. 3770-3780 (see entire document)	1-4, 25-27, 29-33, 39 <u>5-24, 28, 34-</u> <u>38, 40-42</u>
A	Intervirology, Vol. 36, 1993, P.S. Reddy <u>et al.</u> , "Restriction Endonuclease Analysis and Molecular Cloning of Porcine Adenovirus Type 3" pp. 161-168 (see entire document) [cited in application]	1-42

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/AU 98/00648

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report				Patent Family Member			
WO	97/20036	AU	71325/96	ES	1032067	ES	2105984
AU	72646/94	WO	95/02697	EP	667912	FI	951138
		NO	950939	SK	312/95	AU	69052/98
		CA	2144040	CN	1113390	CZ	9500639
		FR	2718749	HU	72558	NZ	269156
		PL	308122				
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WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : A61K 39/235, C12N 15/63, 15/67, 15/86		A1	(11) International Publication Number: WO 99/08706
			(43) International Publication Date: 25 February 1999 (25.02.99)
(21) International Application Number: PCT/AU98/00648		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).	
(22) International Filing Date: 14 August 1998 (14.08.98)		<p>Published With international search report.</p>	
(30) Priority Data: PO 8560 14 August 1997 (14.08.97) AU			
(71) Applicants (for all designated States except US): COMMON-WEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANISATION [AU/AU]; Limestone Avenue, Campbell, ACT 2601 (AU). PIG RESEARCH DEVELOPMENT CORPORATION [AU/AU]; Computer Associate House, 10 National Circuit, Barton, ACT 2600 (AU).			
(72) Inventors; and (75) Inventors/Applicants (for US only): JOHNSON, Michael, Anthony [AU/AU]; 46 Harold Street, Thornbury, VIC 3071 (AU). HAMMOND, Jeffrey, Michael [GB/AU]; 5 Cantala Drive, Jan Juc, VIC 3228 (AU).			
(74) Agent: WATERMARK PATENT & TRADEMARK ATTORNEYS; 2nd floor, 290 Burwood Road, Hawthorn, VIC 3122 (AU).			
(54) Title: RECOMBINANT PORCINE ADENOVIRUS VECTOR			
(57) Abstract			
<p>This invention relates to a recombinant vector including a recombinant porcine adenovirus, stably incorporating and capable of expression of at least one heterologous nucleotide sequence. The nucleotide sequence is preferably one which encodes an antigenic determinant of Hog Cholera Virus or Pseudorabies virus. The further invention relates to a method of production of recombinant vectors, to methods of preparation of vaccines based on the vectors, to administration strategies and to methods of protecting pigs from disease.</p>			

* (Referred to in PCT Gazette No. 32/2000, Section II)

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